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Signed Andrew Gerry

Dated 5 October 2004

Rule 4(1)

Patents Form 1/77

[redacted] Act 1977
6)

The Patent Office

1/77

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

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Cardiff Road
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- 3 OCT 2003

030CT03 E841908-1 D00351
P01/7700 0.00-0323171.9

1. Your reference — DCS/P96074UK

2. Patent application number *(The Patent Office will fill in)* — 0323171.9

3. Full name, address and postcode of the or of each applicant *(underline all surnames)* — Coventry University
Priory Street
Coventry
CV1 5FB

Patents ADP number *(if you know it)* — 08153480001

If the applicant is a corporate body, give the country/state of its incorporation — UK

4. Title of the invention — Fluorescent Compound

5. Name of your agent *(if you have one)* — **Marks & Clerk**

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)* — **144 New Walk**
Leicester
LE1 7JA

Patents ADP number *(if you know it)* — ~~711002~~ 08691164001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'yes' if:*
a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	9
Claim(s)	3
Abstract	0
Drawing(s)	0

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translation of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	One
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature

Date
2 October 2003

12. Name and daytime telephone number of person to contact in the United Kingdom

STAGG, Diana Christine
(0116) 233 8181

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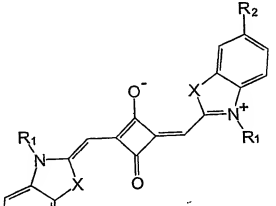
Fluorescent Compound

The present invention relates to novel photoluminescent compounds, to a novel protein detector and to a novel method of detecting the presence of protein in a fluid, in particular water, or bodily fluids.

Photoluminescent compounds are known, and known photoluminescent compounds include the class of dyes known as squaraine dyes, in particular the indolenine series of squaraine dyes, which are substituted and unsubstituted compounds of the general formula:



where X = S, Se or $C(CH_3)_2$
and R_1 = alkyl
and R_2 = H, alkyl or halogen

Known indolenine squaraine dyes include both symmetrical and asymmetrical compounds, and are described, for example, in an article by E. Terpetschnig et al. in Anal. Chim. Acta 282 (1993) pages 633 - 641.

These dyes are insoluble in water and are used, inter alia, for the qualitative detection of the presence of protein in fluids. In order to use these dyes for the detection of the presence of proteins, which are themselves water-soluble in a fluid, in particular an aqueous fluid, the dye has to be dissolved in a solvent comprising a mixture of water and an alcohol such as methanol. When protein is added to a water/methanol solution of an indolenine squaraine

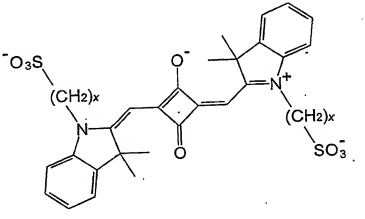
dye, there is an increase in fluorescence. The analytical method disclosed in the article cited above is a purely qualitative method, simply recording the presence or absence of protein in the test fluid sample, and cannot therefore be used to determine the amount of protein present.

Current methods for the quantitative analysis of protein in fluids and semiquantitatively / quantitatively as stains for proteins in gels include the Bradford [M.M. Bradford in Anal. Biochem. 72 (1976) 248], Lowry [O.H. Lowry et al. in J. Biol. Chem. 193 (1951) 265] methods and bicinchoninic acid (BCA) [P.K. Smith et al. in Anal. Biochem. 150 (1985) 76] methods for fluids and silver stain [C.R. Merril in Meth. Enzymol. 182 (1990) 477] and Coomassie Blue [S. Fazekas de St. Groth et al. in Biochim. Biophys. Acta 71 (1963) 377] for gels. The Bradford, Lowry and BCA methods are colourimetric techinques (ie. colour change) and are suitable for solution protein ranges of 8 – 2000 μg/mL, require multiple reagents and incubation times and suffer from interference from numerous common reagents. Coomassie Blue, used in the Bradford method, takes 20 – 30 mins and is not hindered by tris buffers. Silver stain is a very sensitive (10 – 100 ng/mL) chromatogenic-technique and is used as a gel stain but care must be taken in the handling of the gel and the whole technique takes 2 – 3 hours. Coomassie Blue is also a gel stain and can be used instead of silver stain if the protein range is in the chromatogenic μg range. Other commercial techniques for the detection of protein concentration include the NanoOrange™ Protein Quantitation Kit and the CBQCA™ Protein Quantitation Kit, both from Molecular Probes, which are ultra-sensitive solution techniques reliant on changes in fluorescence but are not linear over the ranges given for the squaraine derivative. These two techniques also require 30 mins preparation time.

It is an object of the present invention to provide a novel photoluminescent compound, in particular a novel photoluminescent compound that is soluble in water, or water containing solvent mixtures, and retains its photoluminescent properties in these media.

It is a further object of the present invention to provide a method for detecting the presence of protein in fluids, or as a gel stain, in which the above time constraints are reduced or substantially obviated and at a greater sensitivity.

The present invention provides photoluminescent compounds of the formula



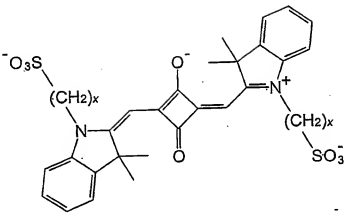
in which x may represent any integer, and in which the phenyl rings may be substituted.

Particularly preferred compounds of the general formula include those wherein $x = 3$.

Further preferred compounds of the general formula include those which are unsubstituted or in which one or both phenyl rings are substituted in the 5-position with alkyl (including ^{i}propyl and ^{t}butyl) or halogen groups.

Particularly preferred compounds of the general formula include 2,4-bis(1-(propan-3-sulfonic acid)-3,3-trimethyl-2-indolinylidenemethyl)cyclobutenediylium-1,3-diolate or any metal or quaternary nitrogen (ie. ammonium, mono-, di- or trialkylammonium, pyridinium etc) salt of the sulfonic acid.

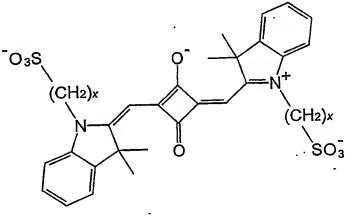
The present invention further provides a protein detector, which comprises a compound of the general formula



in which x may represent any integer, and in which the phenyl rings may be substituted, in water or a water containing solvent mixture, at concentrations from 1×10^{-9} to 1 moles per litre.

The present invention further provides a method for measuring the total dissolved protein content of a fluid sample, which method includes the steps of:

(a) dissolving a compound of general formula;



in which x may represent any integer, and in which the phenyl rings may be substituted, in water or a water containing solvent mixture, at concentrations from 1×10^{-10} to 1 moles per

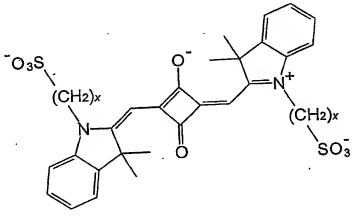
litre.

(b) admixing the solution of step (a) with a test fluid sample;

(c) measuring the fluorescence of the sample; and

(d) comparing the fluorescence with a standard value or values (ie. calibration plot), to obtain a value for the total protein content.

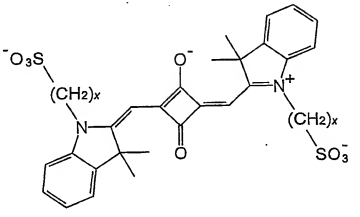
The present invention further provides a method for detecting and / or quantifying proteins separated electrophoretically in a supporting matrix, for example polyacrylamide, agrose or starch, either in the presence or absence of sodium dodecylsulfate (SDS), that has been fixed in an aqueous / organic / acid mixture comprising aqueous methanol and acetic acid, wherein the matrix is subsequently stained with a photoluminescent compound of the general formula;



in which x may represent any integer, and in which the phenyl rings may be substituted, in solution in 10% aqueous methanol or aqueous acetic acid, at a concentration of from 1 x 10^{-10} to 1 moles per litre, and destained to visualise bands.

In order to establish standard values for the fluorescence of aqueous solutions of proteins over the required detection ranges, the fluorescence of solutions containing

known concentrations of the protein BSA, was measured using a 5×10^{-8} M aqueous solution of the compound of formula:



where $x = 3$.

Dye 1

at an excitation wavelength of 624 nm ($\varepsilon = 1.0 \times 10^5$ $mol^{-1}cm^{-1}$) and a detection wavelength of 638 nm.

The results, for three separate calibration plots, over the detection range of 0-500 ng/mL BSA are shown in Figure 1 and the results, for three separate calibration plots, over the detection range of 0-10 μg/mL BSA are shown in Figure 2.

It is particularly striking to note the linear response over these detection ranges.

It is also an advantage of the photo luminescent compounds of the present invention, that once a calibration curve has been constructed using BSA then any number of protein concentrations can be determined instantaneously. The fluorescence is also at a high wavelength and is not masked by any diffuse lower wavelength organic fluorescence.

Examples

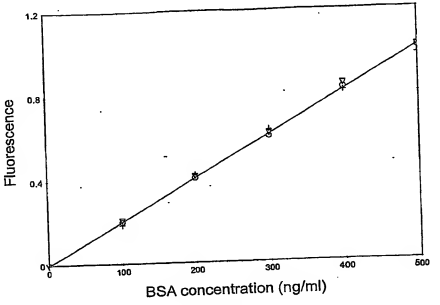


Figure 1

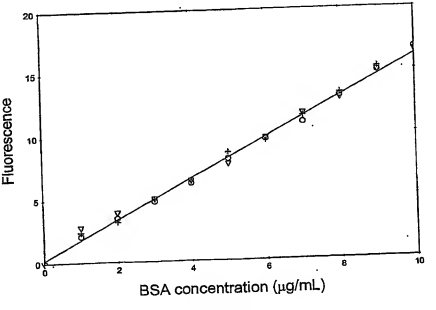
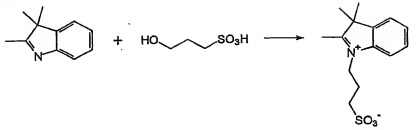


Figure 2

Experimental

Starting materials



2,3,3-trimethyl-1-(propan-3-sulfonyl)indolenine was prepared using the literature method of E. Havinga et al. in Synthetic Metals 69 (1995) pages 581-582 by heating 2,3,3-trimethylindolenine in excess 1,3-propanesultone and toluene, using a Dean and Stark apparatus. Upon cooling the excess solvent was decanted off and the product washed repeatedly with petroleum ether to yield a red oil. Crystals of 2,3,3-trimethyl-1-(propan-3-sulfonyl)indolenine formed from the oil upon standing for several weeks and the single crystal x-ray structure is shown in Figure 3.

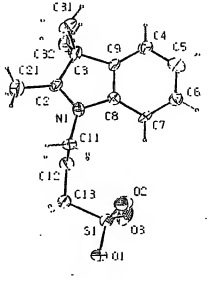
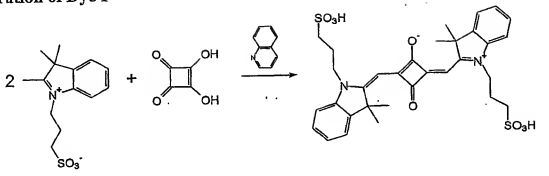


Figure 3

Molecular conformation and atom-naming scheme for the structure of 2,3,3-trimethyl-1-(propan-3-sulfonyl)indolenine.

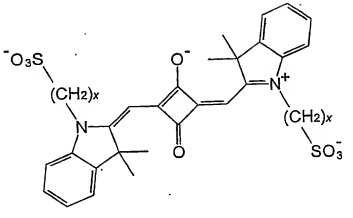
Preparation of Dye 1



2,4-bis(1-(propan-3-sulfonic acid)-3,3-dimethyl-2-indolinylidenemethyl)cyclobutenebis(ylium)-1 3-diolate was prepared using the literature method of Sprenger and Ziegenbein in Agnew. Chem. Int. Ed. 6 (1967) page 533 by refluxing 2:1 molar amounts of 2,3,3-trimethyl-1-(propan-3-sulfonyl)indolenine and squaric acid with catalytic amounts of quinaline in 50/50 toluene/butan-1-ol using a Dean and Stark apparatus. The product was collected in vacuo after removal of the reaction solvents and repeated heated washings with petroleum ether.

Claims

1. A photoluminescent compound of the formula



in wherein x may represent any integer, and in which the phenyl rings may be substituted.

2 A photoluminescent compound according to claim 1 wherein $x = 3$.

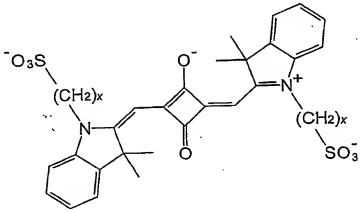
3 A photoluminescent compound according to claim 1 or claim 2 which is unsubstituted.

4 A photoluminescent compound according to claim 1 or claim 2 in which one or both phenyl rings are substituted in the 5-position with alkyl, including ^{i}propyl and ^{t}butyl, or halogen groups.

5. A photoluminescent compound according to any of claims 1 to 4 which is 2,4-bis(1-(propan-3-sulfonic acid)-3,3-trimethyl-2-indolinylidenemethyl)cyclobutenediylium-1,3-diolate or any metal or quaternary nitrogen (ie. ammonium, mono-, di- or trialkylammonium, pyridinium etc) salt of the sulfonic acid.

6. A photoluminescent compound substantially as herein described and with reference to the Examples.

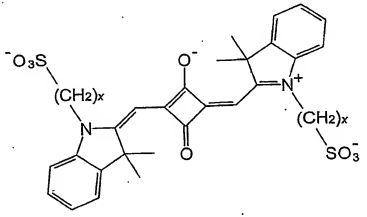
7. A protein detector, which comprises a photoluminescent compound of the general formula



in which x may represent any integer, and in which the phenyl rings may be substituted, in solution in water, at concentrations from 1 x 10^{-10} to 1 moles per litre.

8. A method for measuring the total dissolved protein content of a fluid sample, which method includes the steps of:

(a) dissolving a photoluminescent compound of the general formula;



in which x may represent any integer, and in which the phenyl rings may be substituted, in

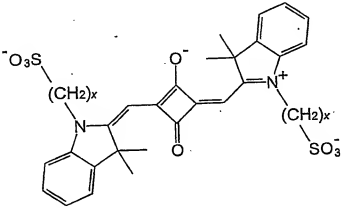
solution in water, at concentrations from 1 x 10^{-10} to 1 moles per litre.

(b) admixing the solution of step (a) with a test fluid sample;

(c) measuring the fluorescence of the sample; and

(d) comparing the fluorescence with a standard value, to obtain a value for the total dissolved protein content.

9. A method for detecting and / or quantifying proteins separated electrophoretically in a supporting matrix, either in the presence or absence of sodium dodecylsulfate (SDS), that has been fixed in an aqueous / organic / acid mixture comprising aqueous methanol and acetic acid, wherein the matrix is subsequently stained with a photoluminescent compound of the general formula;



in which x may represent any integer, and in which the phenyl rings may be substituted, in solution in 10% aqueous methanol or aqueous acetic acid, at a concentration of from 1 x 10^{-10} to 1 moles per litre, and destained to visualise bands.

10. A method according to claim 9 wherein the supporting matrix is polyacrylamide, agrose or starch.

PCT 02/4167